Protoplasts from Plant Materials

Properties of Protoplasts Released by Anaerobic Fermentation with Clostridium roseum

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The 2,000,000 tons of leaf waste incident to the commercial production of vegetable crops contain 50,000 tons of protein and 20 tons of carotene, besides other lipoid constituents. As a preliminary to the recovery of these materials, some method of concentration is desirable. It can be accomplished by release of the protoplasts as a result of a 2-day fermentation of the cell walls. The dried protoplasts contain from 31. to 56% protein, 18 to 27% lipoids, and 0.04 to 0.2% carotene, representing a two- to seven fold increase in concentration over the original leaves. The protein of leaf protoplasts was digestible by trypsin. The process is also applicable to fleshy tissue such as carrot roots, sweet potato tubers, and winter squash. Concentrates containing up to 2.2% carotene were prepared from carrots without solvent extraction.

WHEN cryptostegia leaves (*C. grandiflora*) were subjected to anaerobic fermentation by *Clostridium roseum* as a pretreatment in recovery of rubber, the cell walls were digested and the cell contents (protoplasts) liberated (*4, 7*). The protoplasts remained as discrete entities and could readily be separated from the bagasse (cuticle, ribs, vascular tissue). Since the fractionation of the leaves was so clean cut and the recovery of the protoplasts and of their water-insoluble constituents so high, it was deemed advisable to apply the fermentation process to other plant materials as a first step in the preparation of proteins and lipoids. This paper reports the successful preparation of protein, fat, and carotene concentrates from leaves of turnip, lettuce, beet, grass, and broccoli; from whole pea and snap bean vines and alfalfa; from the roots of carrots and sweet potatoes; and from the fruit of winter squash. Because of the special interest of this laboratory in the utilization of the leaf wastes incident to the production and processing of vegetables, the leaves chosen for

study were mostly from this source. The 2,000,000 tons of such fresh waste produced annually in the United States (*6*) contain 50,000 tons of protein and 20 tons of carotene. Any method for recovering that protein and carotene is intriguing.

The preparation of protein concentrates from grass has been attempted with some success. A mechanical process patented in England (*8*) recovered only part of the proteinaceous material. Sullivan (*9, 10*) dissolved the proteins with dilute alkali and reprecipitated them with acid. The crude product contained 58% protein, which was 56% of the total protein in the grass.

Surplus and cull carrots are the predominant source of commercial carotene. Many present methods for the preparation of carotene necessitate drying the fresh roots in order to make the carotene accessible to nonaqueous solvent extraction. This drying is costly and involves considerable loss of carotene by oxidation. Holmes and Leicester (*3*) have proposed dehydrating the roots with acetone prior to solvent extraction. This method eliminates the oxidative destruction of carotene, but the large quantities of solvent involved make the process expensive. Barnett (*2*) developed a method of recovering a carotene concentrate from fresh carrots by mechanical means. This method requires extensive disintegration of the plant tissues. In fact, unless the walls of the individual cells are broken to allow the escape of the carotene-containing particles, the yield of carotene is low; furthermore, a considerable amount of pulp is incorporated into the concentrate, resulting in much lower carotene purity.

FERMENTATION OF LEAFY VEGETABLE WASTES

Leaf material from eight species of plants was used in this study to determine the applicability of the fermentation process for the recovery of protoplasts. The source, degree of maturity, and portions of plants used in the experiments were as follows:

TABLE I. COMPOSITION OF PLANT MATERIALS AND RECOVERY OF CONSTITUENTS IN THEIR FERMENTATION PRODUCTS

Plant Fraction	Chemical Analysis[a]					Recovery in Products, % of Original				
	Dry wt. g.	Ether-soluble material %	True protein %	Ash %	Carotene[b] γ/g	Dry wt.	Ether-soluble material	True protein	Ash	Carotene
Pea Vines										
Original	1025	3.5	13.2	15.1	118	(100)	(100)	(100)	(100)	(100)
Bagasse	206	4.8	12.7	4.8	24	20.0	28	19.3	6.4	4.1
Protoplasts	147	24.3	35.4	16.0	692	14.3	100	38.6	15.2	84
Snap Bean Vines										
Original	690	3.7	12.7	11.7	155	(100)	(100)	(100)	(100)	(100)
Bagasse	181	3.5	7.4	5.7	39	26.2	24.9	15.3	12.8	6.6
Protoplasts	108	20.5	39.6	17.5	908	15.9	86.7	48.8	23.4	92
Alfalfa										
Original	727	4.1	12.4	6.8	116	(100)	(100)	(100)	(100)	(100)
Bagasse	373	4.6	12.4	2.6	143	51.3	57.7	51.3	19.6	63.2
Protoplasts	97.0	17.9	52.4	2.7	1460	13.3	58.0	56.3	5.2	168
Bluegrass (Lawn Clippings)										
Original	722	9.5	24.8	9.9	234	(100)	(100)	(100)	(100)	(100)
Bagasse	207	8.9	21.4	5.3	116	28.7	26.5	24.7	15.1	14
Protoplasts	140	23.0	55.8	1.6	375	19.3	46.8	43.7	3.1	31
Turnip Tops										
Original	381	3.7	13.0	21.0	229	(100)	(100)	(100)	(100)	(100)
Bagasse	14.4	11.5	13.9	15.4	54	3.8	11.8	4.0	2.8	0.8
Protoplasts	83.0	22.3	31.4	29.2	848	21.8	131	52.7	26.4	81
Beet Tops										
Original	716	6.5	13.2	27.6	220	(100)	(100)	(100)	(100)	(100)
Bagasse	97.6	8.8	21.2	9.5	212	13.6	18.6	21.8	4.7	13
Protoplasts	183	22.1	33.8	15.6	755	25.5	86.7	65.3	14.4	88
Lettuce										
Original	486	10.3	12.0	15.8	126	(100)	(100)	(100)	(100)	(100)
Bagasse	34.3	10.2	7.0	5.7	49	7.0	7.0	4.1	2.5	0.2
Protoplasts	58.1	26.6	38.3	12.7	490	13.1	30.8	38.1	9.6	46
Broccoli										
Original	746	5.8	17.5	13.3	469	(100)	(100)	(100)	(100)	(100)
Bagasse	22.5	32.5	10.4	2.3	102	3.0	17.0	1.7	0.6	0.6
Protoplasts	295	21.8	54.3	2.0	2000	39.5	149	118	5.9	168

[a] Moisture-free basis.
[b] In adsorption of pigment solution from fermentation products in Wall and Kelley procedure, several additional bands developed; these were included as "carotene," which may mean that the values for carotene after fermentation are too high. This phenomenon was confined to carotene determination on fermentation products of leafy materials and was not noted in products reported in Table III.

The fermentations were complete in 2 days. The tissues were then disintegrated by mild agitation, and the released protoplasts were separated from the fibrous materials by wet screening through a combination of 20- and 80-mesh sieves. Although on a large scale an 80-mesh gyrating screen alone was adequate, on a laboratory scale the separation was facilitated by the use of a coarse screen followed by a fine one.

The residue, bagasse, was resuspended in a small volume of water and screened again to recover mechanically trapped protoplasts and then dried in circulating air at 34° C.

The protoplasts, which settled to one-fifth volume or less overnight, were washed twice by decantation, concentrated to a thick paste by centrifuging, and dried in circulating air at 34° C.

The following analyses were made on the original plants and on fermentation products:

Pea vines. From garden-grown, fully mature plants with many dying leaves. The whole vine, minus roots and pods.

Snap beans. Garden-grown, fully mature, whole plants, minus roots and pods.

Alfalfa. Field-grown plants just starting to bloom. Dead and woody stalks discarded.

Bluegrass. 2-Inch clippings from a lawn consisting predominantly of bluegrass; very lush growth collected in May, air-dried at 34° C., and stored at 30–34° C. for 3 months before use.

Turnip tops. Leaves, including petioles, from garden-grown, mature plants.

Beet tops. Leaves, including petioles, from farm-grown, mature plants.

Lettuce. Leaves from garden-grown plants which had bolted and were starting to blossom.

Broccoli leaves. Webs stripped from midribs of farm-grown plants in the edible stage.

It was found that the fermentation procedure developed for recovery of cryptostegia protoplasts (7) could be applied directly or with only slight modification to these materials. It was unnecessary to preboil them, since, unlike cryptostegia leaves, none contained substances inhibitory to *C. roseum*. It was deemed advisable, however, to blanch the tissues by steaming to destroy enzyme activity and to wilt the leaves so that they would pack more readily into the fermenter.

The leaves were first rinsed several times with water to remove the adhering soil and sand particles. They were cut into 2- to 3-inch (5- to 7.5-cm.) pieces, packed in a 22-liter round-bottomed flask, steamed for 10 minutes at atmospheric pressure, and cooled. They were then diluted with tap water to a concentration of 5% solids, calculated on the dry weight of the original leaves, and inoculated with 10% volume of an 18-hour broth culture of *C. roseum* (McCoy,

Moisture was determined by heating at 65° C. in vacuo to constant weight. Total nitrogen was determined by the Kjeldahl procedure on ether-extracted material (to exclude lipide and other ether-soluble nitrogen) and calculated to the original dry basis. To determine the nonprotein nitrogen, 2 grams of dried product were treated with 80 ml. of a 5% solution of trichloroacetic acid, heated to boiling, and filtered into a 100-ml. volumetric flask. The filtrate was made up to volume by continued washing of the residue with 5% trichloroacetic acid, and the nitrogen was determined by the Kjeldahl method. The difference between total nitrogen of the ether-extracted material and the nonprotein nitrogen was considered to be the true protein nitrogen, which, multiplied by 6.25, gave the true protein. Ash, ether extract, and crude fiber were determined according to official methods (1). Carotene was determined by the method of W and Kelley (12). The results are shown in Table I.

DIGESTION OF PROTOPLASTS BY TRYPSIN

With the possibility in mind of using the protoplasts in feed, it was deemed advisable to demonstrate their digestion by proteolytic enzymes. To protoplasts (40 mg. of protein nitrogen or 1.82 mg. of nitrogen per ml. in final volume) suspended in 10 ml. of 0.1 *M* phosphate buffer, pH 8.8, were added 2 ml. of trypsin solution (1 gram of commercial trypsin in 50 ml. of 0.1 *M* phos-

phate buffer, pH 8.8). To each were added 4 drops of toluene as a preservative. The enzyme solution, incubated for a similar period, was added to the controls at the end of the incubation period. After incubation at 34° C. for 24 hours, 10 ml. of 10% solution of trichloroacetic acid were added to each, the mixtures were filtered, and nitrogen in the filtrates was determined. Table II shows that under these conditions the protoplasts are susceptible to digestion by trypsin.

FERMENTATION OF ROOTS AND FRUITS

In contrast to the cells in leaf tissue, those of storage tissue, such as the ones in Table III, are highly vacuolated, with masses of carbohydrates and oils, so that the proteinaceous phase is not so continuous as it is in the leaf chlorenchyma. Destruction of the cell walls and removal of the carbohydrates by fermentation and solution resulted in fragmented undefined protoplasts rich in oil. Microscopic examination of the material from carrots owed free oil globules and carotene crystals.

Furthermore, the high carbohydrate content of the storage tissues led to formation of considerable gum. This appeared as a viscous, slimy layer full of gas bubbles, which floated on the surface of the fermentation liquor. The protoplast fraction, together with the skins and unfermented cellulosic materials, was trapped within this gum, from which it was separated with some difficulty.

CARROTS. Fresh carrots were ground fine and extracted once with boiling water to remove excess carbohydrates and hence to reduce the amount of slime formation. The extracted plant material was then made up to 5% solids, inoculated with an 18-hour broth culture of C. roseum, and covered with nitrogen. Incubation was carried out at 34° C. for 2 days. By this time the fermentation was complete, and the material, reduced considerably in volume, had formed a frothy layer on the surface. The liquor was drained off, and the gummy layer was diluted with an equal quantity of water, made neutral with sodium hydroxide, treated with 0.03% (weight by volume) of aluminum sulfate to facilitate subsequent flocculation, boiled, and passed through an 80-mesh screen. The bagasse (outer cork tissues and the central xylem) stayed on the screen; the carotene-containing oil globules and a small quantity of adhering protoplasts passed through. The proteinaceous material flocculated on cooling, and when it settled carried down the carotene-containing oil globules. The coagulum was recovered and dried. Seven experiments on the preparation of a carotene concentrate from carrots were carried out, with analytical control. The results are shown in Table III.

SWEET POTATOES. Sweet potatoes obtained on the market were ground in a meat grinder, and 1 kg. of the pulp (252 grams, v weight) was boiled for 15 minutes with 1.5 liters of water and ained. A preliminary experiment indicated the necessity of preboiling the pulp. After cooling, the pulp was inoculated with 200 cc. of an 18-hour broth culture of C. roseum, the volume made up to 2 liters, covered with nitrogen, and incubated at 37° C. for 4 days, although the fermentation was almost complete in 2 days. At the end of fermentation, about 400 cc. of a slimy, yellow-brown, gummy layer were on the surface, containing the skins and other debris. The liquid, underlying this was clear but orange colored. The upper layer was recovered and diluted with 300 cc. of water, neutralized, treated with 3 grams of aluminum sulfate, and boiled for 20 minutes. Screening removed 2.2 grams, dry weight, of undigested corky tissue. The solids flocculated and

after settling overnight had compacted to a small volume. This was further concentrated by centrifuging and dried at 50° C. in vacuo. Table III shows that a sixteen fold increase in carotene concentration was obtained.

SQUASH. Winter squash (Hubbard) was ground in a meat grinder, and 1 kg. (167.8 grams, dry weight) of the pulp was boiled for 20 minutes in 1.5 liters of water and drained on cheesecloth. The pulp was inoculated with 250 cc. of an 18-hour culture of C. roseum, made up to 2 liters, covered with nitrogen, and fermented at 37° C. To determine the effect of excess soluble carbohydrate, 1 kg. of pulp was fermented without hot-water extraction. When the fermentations were complete, the pulps had been reduced in amount and had risen to the surface as a gummy layer. The boiled pulp yielded a larger volume of a more slimy gum than the unboiled. The gummy layers were diluted with water to 1.5 liters, neutralized, treated with 3 grams of aluminum sulfate, and boiled for 20 minutes. They were then screened to remove coarse particles of corky tissue and set aside to allow the proteinaceous material to flocculate. The material from the extracted pulp did not flocculate until 1.5 grams more of aluminum sulfate were added and it was boiled again. The supernatants were decanted and the sediments centrifuged. The resulting concentrates were then dried in vacuo at 50° C. Analytical results are shown in Table III.

DISCUSSION

All plants fermented rapidly, loss in dry weight ranging from 35% in alfalfa to 80% in lettuce. Plants containing a large proportion of stems and fibers (pea vines, bean vines, alfalfa, and grass) showed less loss in weight than those consisting mostly of leaves and yielded more bagasse (20 to 51%). The authors have observed that C. roseum does not attack the cellulose of the vascular system, which comprises the greater part of stems and veins (7). Photomicrographs in that publication show this type of disintegration.

The protoplast fraction, which represented from 13 to 25% of the weight of the original material, was rich in protein (31 to 56%), ether-soluble materials (18 to 27%), and carotene (0.04 to 0.20%). This concentration was accomplished by the extensive digestion of carbohydrate and cellulosic materials during fermentation and also by the removal by the screening process of unfermented gross structures (cuticle, vascular tissue, etc.). Lettuce leaves and lawn clippings gave low yields of protoplasts, protein, and carotene, probably because the protoplasts became highly fragmented during the fermentation, did not sediment properly, and consequently were not completely recovered.

The data of Table I show that ether-soluble substances were recovered in fairly good yields from most of the fermented plants. The ether extracts of the protoplasts were usually high. Recoveries above 100% may have been due to the absorption of compounds produced in the fermentation, or possibly to the greater availability of certain constituents to extraction, since, in most cases in which the recovery of ether-soluble substances was more than 100%, that of carotene was also high. In the case of cryptostegia leaves (4), rubber could not be completely removed from untreated leaves even after prolonged extraction. After fermentation, however, the acetone-soluble constituents and the rubber were extracted rapidly and completely.

Except for alfalfa and broccoli, protein yields were somewhat low. Loss of protein in the process could be caused by three factors: incomplete recovery of the protoplasts during washing, mechanical entrapment of protoplasts in the bagasse fraction, and destruction of protein by the organism. The first loss is dependent on the nature of the plant material; bluegrass and lettuce yielded fragmented protoplasts with poor settling properties. Bluegrass and alfalfa bagasse, which was fibrous, entrapped the protoplasts. The contribution of the third factor was not determined; in two fermentations, alfalfa and broccoli, all the original protein was accounted for in the protoplasts and bagasse. Both these plants yielded relatively large protoplasts, which settled rapidly, with consequent negligible loss of fragmented protoplasts in the wash water. However, no mechanical loss was apparent in turnip tops, beet tops, pea vines, and bean vines, and

TABLE III. CAROTENE CONCENTRATES OBTAINED BY FERMENTATION OF ROOTS AND FRUITS

No.	Plant Fraction	Dry Weight		Carotene		
		G.	%	Analysis, γ/g.	Total, γ	% of Orig.
	Carrots					
14B	Original	839	100	1,140	956,000	100
	Bagasse	38.8	4.6	1,120	43,400	4.5
	Concentrate	42.0	5.0	22,400	941,000	98.5
	Processing liquor	...	...	...a		
17A	Original	120.4	100	870	105,000	100
	Bagasse	1.7	1.4	810	1,400	1.3
	Concentrate	6.5	5.4	15,500	101,000	97.1
	Processing liquor	2140	...	13.4b	28,700	27.3
19A	Original	134	100	728	97,500	100
	Bagasse	1.5	1.1	960	1,440	2.4
	Concentrate	11.3	8.4	13,160	148,700	152
	Processing liquor	2060	...	1.4	2,880	2.9
22B	Original	2960	100	625	1,850,000	100
	Bagasse	26.7	0.9	940	25,100	1.3
	Concentrate	190.7	6.4	9,312	1,778,000	90.6
	Processing liquor	...	...	...a		
24A	Original	439	100	778	339,000	100
	Bagasse	8.30	1.9	1,220	9,950	2.9
	Concentratec	14.91	3.4	11,200	167,000	49.3
	Processing liquor	2450	...	1.8	4,420	1.3
25A	Original	236	100	778	183,600	100
	Bagasse	3.48	1.5	415	1,450	0.8
	Concentrate	12.30	5.2	6,800	83,700	45.5
	Processing liquor	...	...	...d		
26A	Original	236	100	778	183,600	100
	Bagasse	4.48	1.9	490	2,190	1.2
	Concentrate	18.80	8.0	6,240	117,200	63.8
	Processing liquor	...	...	...d		
	Sweet Potatoes					
22A	Original	252.0	100	3.2	806	100
	Bagasse	2.2	0.9			
	Concentrate	17.4	6.9	52.0	905	112
	Winter Squash					
23A	Original	167.8	100	140	23,500	100
	Concentrate from unextracted pulp	35.3	21.0	875	30,900	131
	Concentrate from extracted pulp	12.9	7.7	1,830	23,600	100

a Al-treated processing liquor was not colored and hence presumably contained no carotene.
b Color of liquor was orange.
c Additional 1.34 g. of concentrate analyzing 7,050 γ carotene per g. recovered from fermentation liquor in this experiment.
d These liquors were similar in color to that from experiment 24A.

this leads one to suppose that part of the protein was digested by C. roseum and lost as soluble nitrogen. The proximate analysis carried out on the protoplasts accounts for only 70 to 80% of the dry weight. What constitutes the remainder is unknown. Since only the protoplasts constitute the enriched fraction, the percentage of total protein and other constituents recovered in this fraction is of interest. These data are included in Table I. Because of the fibrous nature of alfalfa, it was difficult to make a clean separation of the protoplasts from the bagasse, with the result that only 56% of the original protein was recovered in the protoplast fraction. Beet tops gave a better separation—65%— while broccoli leaves proved to be the best, with complete recovery. It is possible that, by introducing some modification, quantitative recoveries can be obtained from most plants. The true protein in the protoplast fractions of the plants used represents a two- to fourfold concentration over the original plant, with protein contents ranging from 31 to 56%. Extraction of the ether-soluble substances would increase the protein content by at least 20% and at the same time recover the carotene and fats.

The nonprotein nitrogen, which ranged from 0.6% in snap bean vines to 2.9% in broccoli, represents a considerable fraction of the nitrogenous material and one which cannot be recovered by the method described here, as it is noncoagulable and leaches out of the protoplasts.

The crude fiber in the protoplasts, which ranged from 0.8% in broccoli to 5.1% in lettuce, represents a small amount of fine cellulosic debris which passed through the screen along with the protoplasts in the separation process.

A considerable quantity of the ash was leached out during the fermentation and washing of the protoplasts. The high values

obtained in the protoplasts from certain low-growing vegetables (turnips, beets, peas, beans, and lettuce) may be due partly to sand not removed during the washing.

Carotene was recovered in good yield except in lettuce and lawn clippings. Recoveries of over 100% obtained with alfalfa and broccoli may reflect the greater ease of extraction from fermented plants (4), and may also be due to the presence of interfering pigments (see Table I). The protoplast fraction contains most of the carotene and represents a four- to twelvefold concentration, in the case of broccoli yielding a product with almost 0.2% carotene.

Attempts to ferment rhubarb leaves were unsuccessful. The fermentation of unextracted leaves was slow and incomplete, and even after the leaves were extracted three to four times with boiling water the results were unsatisfactory. This was apparently due to the presence of antibiotic substances (5).

Roots (carrot and sweet potatoes) and fruits (squash) were fermented successfully. Since these tissues contain only small quantities of protein and cellulosic materials, they were almost completely digested.

The product from carrots contained 0.9 to 2.2% carotene (Table III) and represented 91 to 98% of the initial carotene content of the carrots except for the last three runs, which are discussed below. The Wall-Kelley carotene procedure occasionally gave erratic results when applied to the concentrates. Possibly the high fat content of these products (20 to 30%) interfered with the chromatographic phase of the determination (13, p. 321). A saponification stop was introduced in the procedure for the last three experiments (24A, 25A, and 26A), and the carotene values of these experiments are believed to be more trustworthy.

The last three experiments were overfermented. They were run at 37° to 40° C. (instead of 34°), with frequent agitation, for 48 hours, by which time gas production had ceased. Overfermentation was indicated by a tendency for part of the fermented material to sink, rather than remain in a coherent slimy layer on the surface, thus making complete recovery of the carrot concentrate impossible. Furthermore, the specific gravity of the particles of the concentrate was so near that of the liquor that recovery by centrifugation was poor. This would indicate that fermentation should be terminated while the material is still in an adherent frothy layer and before the liquor shows color from settling particles. Under the conditions employed in the last three experiments, the fermentation was complete at approximately 30 hours. Carotene analyses of the processing liquors (but not the fermentation liquors) were made when their color indicated the presence of carotene. The carotene crystals in the concentrate probably had existed in the original tissue, since no solvent action was involved.

No statement can be made concerning the effect of the fermentation and recovery process on the carotene, since the extent of modification, if any, of the pigments was not investigated. Further work on this point is desirable.

The products recovered from carrots were 13 to 20 times richer in carotene than the starting materials. The fermentation reduced the dry weight of the carotene-containing materials to 5%, producing as much as a twenty fold increase in carotene concentration, with a resulting carotene content of 0.9 to 2.2%. The advantages of such concentration before solvent recovery of the carotene are obvious. Such a concentrate would be of practical use without further treatment, but the effect of the fermentation on the quality and biological value of the carotene produced should be carefully investigated.

Fermentations of squash and sweet potato were satisfactory, the carotene being concentrated from 13 to 16 times, but the low initial level of carotene makes fermentation of these materials questionable for this purpose. This sample of sweet potato, however, was unusually low in carotene; many lots contain 25 times as much (11).

Table III shows that 79% of the squash was lost during fermentation. Removal of soluble carbohydrates by preboiling increased the total loss to 92%. Fermentation of the extracted pulp resulted in a thirteen fold concentration of carotene, as compared with a sixfold concentration obtained by fermentation of the unextracted pulp.

Although *C. roseum* can be considered as belonging to the acetone-butanol producing organisms, it is not so efficient in this regard as the acetobutylicum group; hence the carrot and sweet potato starches and sugars are not efficiently utilized. It is not unlikely that an organism can be found that will possess the cellulose-digesting power of *C. roseum* and will also be an efficient solvent producer. No attempt was made to use a mixed culture of a good solvent producer together with *C. roseum*, but such a procedure may be effective.

ACKNOWLEDGMENT

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